CHAPTER 3

RESULTS

3.1 Serum Samples

One hundred and sixty four NPC and 147 non-NPC controls sera were obtained. Of the 164 NPC sera, 127 sera (77.4%) were from males while 37 (22.6%) were from females. The male/female ratio of NPC patients was 3.4:1. The sex distribution of the non-NPC controls was similar to NPC with male/female ratio of 3.2:1 (Table 3.1).

The Ibans with 69 cases made up 42.1% of the NPC samples while Chinese with 37 cases made up 22.5%, the Bidayuhs with 24 samples (14.6%) and Malays (10.4%). The other ethnic groups with smaller number of samples made up the rest of the samples (Table 3.2).

The frequency of NPC patients rose after the age group of 20-29 and reached a peak between 40 and 49 years with 31.1% of the cases from that age group (Table 3.3).

Table 3.1: Distribution of sera by sex

Sex	NPC		Controls	
	No.	%	No.	%
Male	127	77.4	112	76.2
Female	37	22.6	35	23.8
Total	164	100.0	147	100.0

Table 3.2: Distribution of sera by ethnic group

Ethnic group	NPC		Controls	
	No.	%	No.	%
Iban	69	42.1	62	42.2
Chinese	37	22.5	36	24.5
Bidayuh	24	14.6	23	15.6
Malay	17	10.4	19	12.9
Kenyah	5	3.1	2	1.4
Melanau	5	3.1	3	2.0
Kayan	3	1.8	2	1.4
Punan	1	0.6	0	0.0
Saben	1	0.6	0	0.0
Penan	1	0.6	0	0.0
Not available	1	0.6	0	0.0
Total	164	100.0	147	100.0

Table 3.3: Distribution of sera by age group

Age group (years)	NPC		Controls	
	No.	%	No.	%
<20	5	3.1	3	2.0
20-29	13	7.9	16	11.0
30-39	22	13.4	19	12.9
40-49	51	31.1	44	29.9
50-59	38	23.2	28	19.1
60-69	24	14.6	23	15.6
70-79	10	6.1	13	8.8
>80	1	0.6	1	0.7
Total	164	100.0	147	100.0

3.2 Immunofluorescence Assay (IFA) for Anti-EBV Antibodies

Antibody titres for 165 NPC patients and 147 non-NPC controls for IgA/ VCA, IgG/VCA, IgA/ EA and IgG/EA were titrated by IFA (Appendix 3 & 4). Positive samples in the IFA gave the cells a yellowish-green fluorescence under the ultraviolet light microscope (Figure 3.1). On the other hand, cells were homogenous green in colour without distinct fluorescence when reacted with negative samples (Figure 3.2). Similar immunofluorescence patterns were observed for both IgA and IgG of VCA staining on P3HR-1 cells and EA on Raji cells.

3.3 Titres and Distribution of Anti-EBV Antibodies

IgA/VCA titres of <5 to 1280 were observed in NPC sera, with 30.0% showing the most common titre of 40. While in non-NPC controls, titres ranged from <5 to 10 with 85.1% of titre being <5 (Table 3.4 and Figure 3.3). The titres of IgG/VCA ranged from 10 to 10240 in the NPC sera, while in non-NPC controls, titre of 5 to 160 with 55.1% with the titre of 10 was observed (Table 3.5 and Figure 3.4).

IgA/EA titres of NPC sera ranged from <5 to 1280 with 25.0% of samples showing titre <5, while 100% of the non-NPC controls gave the titre of <5 (Table 3.6 and Figure 3.5). IgG/EA titres in NPC sera ranged from <5 to 2560 with 28.0% sera with the titre of 160 while, titres ranged from <5 to 40 with 67.3% showing titre of <5 was observed in non-NPC controls (Table 3.7 and Figure 3.6).

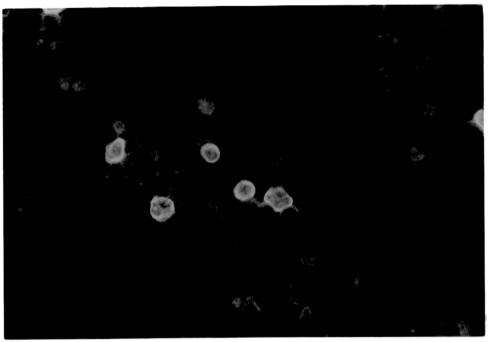

Figure 3.1: Positive immunofluorescence assay (IFA) staining of IgA/EA with Raji cells (Magnification X200).

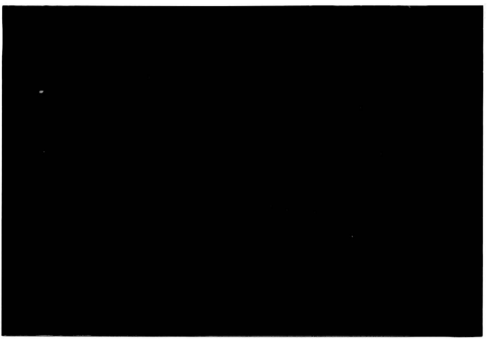

Figure 3.2: Negative immunofluorescence assay (IFA) staining of IgA/EA with Raji cells (Magnification X200).

Table 3.4 : Titres of IgA/VCA in NPC and non-NPC controls

Group	No	Titres						
		<5	5	10	40	160	640	1280
NPC	164	12 (7.3)	15 (9.1)	38 (23.2)	49 (30.0)	31 (18.9)	16 (9.7)	3 (1.8)
Controls	147	125 (85.1)	18 (12.2)	4 (2.7)	0 (0)	0 (0)	0 (0)	0 (0)

% in brackets

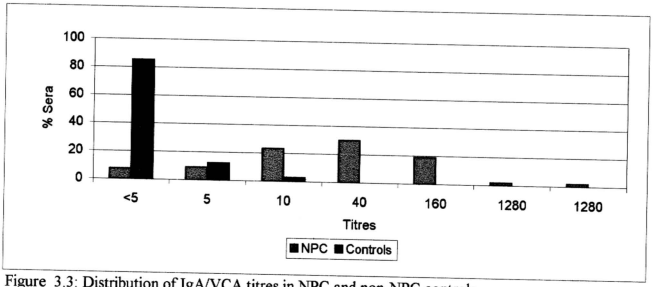

Figure 3.3: Distribution of IgA/VCA titres in NPC and non-NPC controls.

Table 3.5: Titres of IgG/VCA in NPC and non-NPC controls

Group	No	Titres									
		<5	5	10	40	160	640	1280	2560	5120	10240
NPC	164	0 (0)	0 (0)	5 (3.1)	13 (7.9)	48 (29.3)	45 (27.4)	36 (22.0)	14 (8.5)	2 (1.2)	1 (0.6)
Controls	147	0 (0)	46 (31.3)	81 (55.1)	17 (11.6)	3 (2.0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

% in brackets

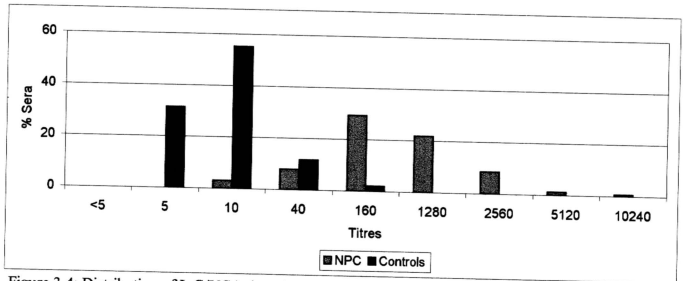

Figure 3.4: Distribution of IgG/VCA titres in NPC and non-NPC controls.

Table 3.6: Titres of IgA/EA in NPC and non-NPC controls

Group	No	Titres						
		<5	5	10	40	160	640	1280
NPC	164	41 (25.0)	18 (11.0)	39 (23.8)	35 (21.3)	25 (15.2)	5 (3.1)	1 (0.6)
Controls	147	147 (100.0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

% in brackets

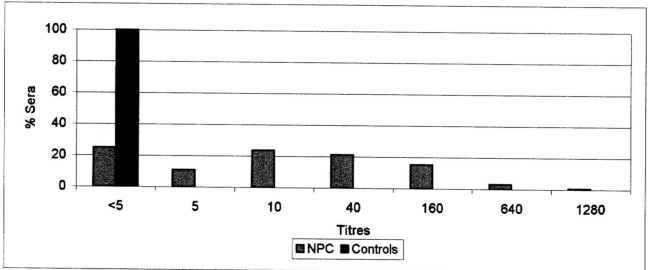

Figure 3.5: Distribution of IgA/EA titres in NPC and non-NPC controls.

Table 3.7: Titres of IgG/EA in NPC and non-NPC controls

Groups	No	Titres							
		<5	5	10	40	160	640	1280	2560
NPC	164	9 (5.5)	9 (5.5)	20(12.2)	41 (25.0)	46 (28.0)	29 (17.7)	9 (5.5)	1 (0.6)
Controls	147	99 (67.3)	22 (15.0)	25 (17.0)	1 (0.7)	0 (0)	0 (0)	0 (0)	0 (0)

% in brackets

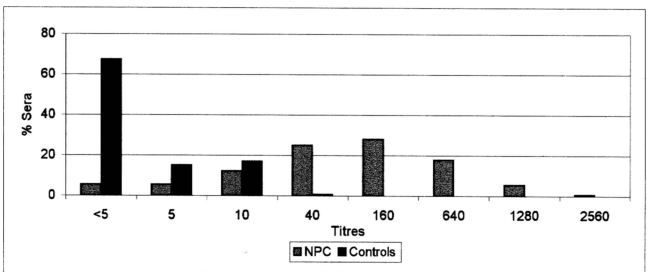

Figure 3.6: Distribution of IgG/EA titres in NPC and non-NPC controls.

The geometric mean titres (GMT) of the titres were calculated using log values of the titres (Appendix 5). A titre of <5 was assigned an arbitrary value of 2. The Mann-Whitney U test (Appendix 5) was used to test for the significance in differences. All 4 markers (IgA/VCA, IgG/VCA, IgA/EA and IgG/EA) showed highly significant differences between NPC and non-NPC controls at the 5% probability level ($p < 0.05$) (Table 3.8).

Table 3.8: GMT and significance values between NPC and non-NPC controls

	GMT		
Marker	NPC	Controls	z-value
IgA/VCA	34.8	2.3	13.55, $p < 0.05$
IgG/VCA	412.4	9.6	14.61, $p < 0.05$
IgA/EA	14.9	2.0	11.39, $p < 0.05$
IgG/EA	76.0	3.1	13.30, $p < 0.05$

3.3.1 Anti-EBV antibodies with respect to sex

The GMT of IgA/VCA, IgG/VCA, IgA/EA and IgG/EA in relation to the NPC patients' sex was analysed (Table 3.9 & Figure 3.7). The anti-EBV antibody patterns were similar in male and female groups, with no significant difference ($p > 0.05$) in the titres analysed by the Mann-Whitney U test (Table 3.9).

Table 3.9: Geometric mean titres (GMT) and p value of anti-EBV antibodies in NPC patients with respect to sex

Sex	No. of Samples	GMT			
		IgA/VCA	IgG/VCA	IgA/EA	IgG/EA
Male	127	38.4	453.8	15.4	93.2
Female	37	24.6	296.9	13.2	37.6
p value		0.13	0.14	0.47	0.08

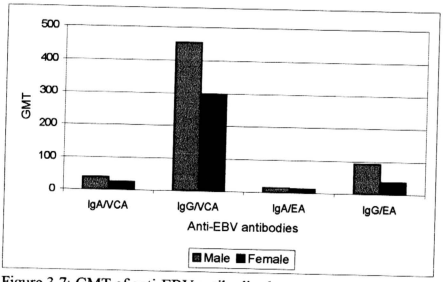

Figure 3.7: GMT of anti-EBV antibodies by sex.

3.3.2 Anti-EBV antibodies with respect to ethnic groups

The GMTs of IgA/VCA, IgG/VCA, IgA/EA and IgG/EA in relation to the NPC patients' ethnic group were analysed (Table 3.10). There is bias in certain ethnic groups due to the limited sample size. The ethnic groups are the Kenyah and the Melanau with 5 samples in each group, the Kayan with 3 samples, Punan, Saben and Penan with 1 sample in each group and 1 sample with unspecified ethnic group. These groups were therefore grouped as 'other indigenous group'. Therefore, for comparison of anti-EBV antibodies with respect to ethnic groups, 5 ethnic groups were considered, i.e. Iban, Chinese, Bidayuh, Malay and others.

The highest GMT value for IgA/VCA (47.8) (Figure 3.8) and IgA/EA (18.7) (Figure 3.10) were in the Malays. Utilising the Mann-Whitney U test, there was no significant difference ($p > 0.05$) between the Malay patients' titres compared to titres of the other 4 ethnic groups. For IgA/VCA, $p = 0.40$ and IgA/EA, $p = 0.59$.

The Bidayuhs showed the highest GMT for IgG/VCA (586.9) (Figure 3.9) and IgG/EA (97.9) (Figure 3.11). Nevertheless, there was no significant difference in the Bidayuh patients' titres of IgG/VCA ($p = 0.24$) and IgG/EA ($p = 0.49$) compared to titres of the other 4 ethnic groups.

Titres of the Chinese and Ibans were also compared and there was no significant difference in the titres of IgA/VCA ($p = 0.38$), IgG/VCA ($p = 0.41$), IgA/EA ($p = 0.45$) and IgG/EA ($p = 0.84$).

Table 3.10:Geometric mean titres (GMT) of anti-EBV antibodies in NPC patients with respect to ethnic groups

Ethnic group	No. of samples	GMT			
		IgA/VCA	IgG/VCA	IgA/EA	IgG/EA
Iban	69	30.8	441.3	13.7	71.1
Chinese	37	39.0	320.0	16.9	78.0
Bidayuh	24	43.2	586.9	14.7	97.9
Malay	17	47.8	320.0	18.7	88.1
Others	17	23.5	425.7	12.5	56.2

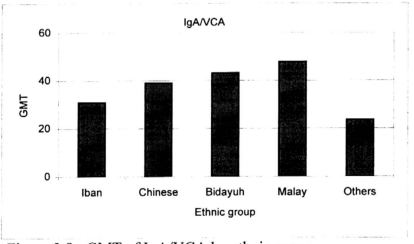

Figure 3.8 : GMT of IgA/VCA by ethnic group.

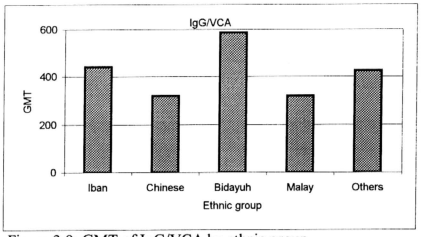

Figure 3.9: GMT of IgG/VCA by ethnic group.

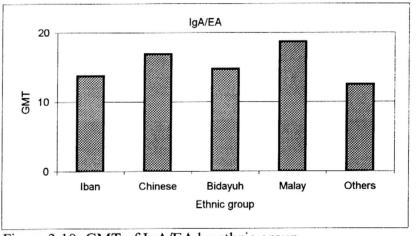

Figure 3.10: GMT of IgA/EA by ethnic group.

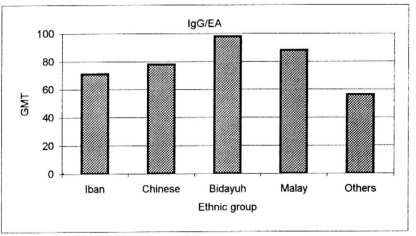

Figure 3.11: GMT of IgG/EA by ethnic group.

3.3.3 Anti-EBV antibodies with respect to age groups

The GMTs of IgA/VCA, IgG/VCA, IgA/EA and IgG/EA in relation to the NPC patients' age were analysed (Table 3.11). Utilising the Mann-Whitney U test, there was no significant difference ($p > 0.05$) in the particular age group's titres where peak was observed compared to the other age groups.

IgA/VCA titres ranged from <5 to 1280 (Table 3.4) with GMT of 16.7 in the <20 years age group. Higher GMT was observed at the age groups 20-29 years (GMT = 57.1) ($p = 0.34$) and 60-69 years (GMT = 48.1) ($p = 0.29$) (Figure 3.12).

IgG/VCA had a wider titres ranged from 10-10240 (Table 3.5). Peaks were also observed at the age group of 20-29 years (GMT = 606.8) ($p = 0.19$) and >80 years (GMT = 640.0) (Figure 3.13). However, this phenomenon may not be the true reflection since there was only 1 patient in the >80 age group.

IgA/EA with the same titre range as IgA/VCA (Table 3.6) showed similar pattern with IgA/VCA with peaks at age groups of 20-29 years (GMT = 19.0) ($p = 0.50$) and 60-69 years (GMT = 18.0) ($p = 0.52$) (Figure 3.14).

IgG/EA titres ranged from <5 to 2560 (Table 3.7) with the GMT ranged of 43.9 to 640.0 (Figure 3.15). An obvious GMT peak was observed in the >80 years age group but the lack of sample in the >80 years (1 sample) may have contributed to the peak.

Table 3.11:Geometric mean titres (GMT) of anti-EBV antibodies in NPC patients with respect to age groups

Age group (years)	No. of samples	GMT			
		IgA/VCA	IgG/VCA	IgA/EA	IgG/EA
<20	5	16.7	422.2	15.9	160.0
20-29	13	57.1	606.8	19.0	95.7
30-39	22	30.8	467.0	15.9	52.6
40-49	51	32.9	397.7	14.6	84.5
50-59	38	37.2	436.3	14.0	78.6
60-69	24	48.1	339.0	18.0	69.9
70-79	10	18.2	259.9	8.2	43.9
>80	1	10.0	640.0	10.0	640.0

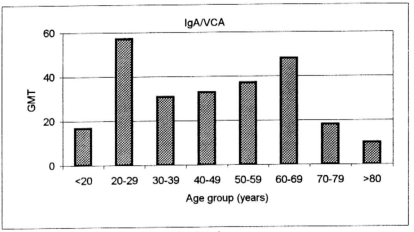

Figure 3.12: GMT of IgA/VCA by age group.

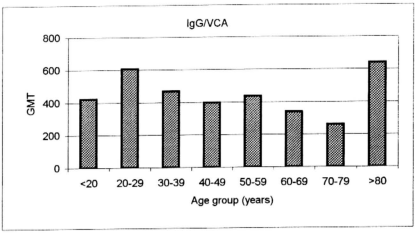

Figure 3.13: GMT of IgG/VCA by age group.

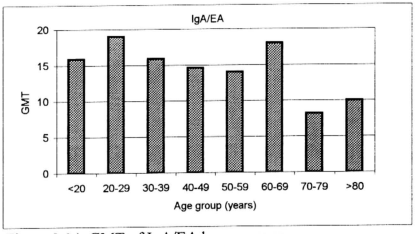

Figure 3.14: GMT of IgA/EA by age group.

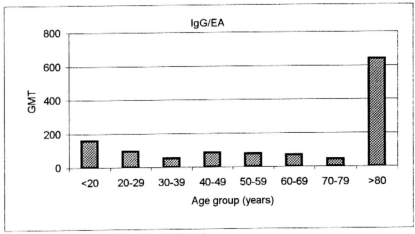

Figure 3.15: GMT of IgG/EA by age group.

3.4 Determination of Cut-off Titres and Geometric Mean Titres (GMT)

The cut-off titre for each marker was determined by considering the sensitivity (%) and specificity (%) at various cut-off titres (Table 3.12). Sensitivity and specificity need to be balanced for a diagnostic marker to be effective. The majority of the control sera gave background staining with both P3HR-1 and Raji cells at titres of 5 to 10 for IgA and 40 to 160 for IgG. From Table 3.12, the cut-off titre for IgA/VCA detection was designated at ≥10, IgG/VCA at ≥160, IgA/EA at ≥5 and IgG/EA at ≥40.

Table 3.12: Comparison of sensitivity and specificity of anti-EBV antibodies at different cut-off titres

Assay	Cut-off titre	Sensitivity[a] (%)	Specificity[b] (%)
IgA/VCA	≥ 5	92.7	85.1
	≥ 10	83.6	97.3
	≥ 40	60.4	100.0
IgG/VCA	≥ 5	100.0	0.0
	≥ 10	100.0	31.3
	≥ 40	96.9	86.4
	≥ 160	89.0	98.0
IgA/EA	≥ 5	75.0	100.0
	≥ 10	64.0	100.0
	≥ 40	40.2	100.0
IgG/EA	≥ 5	94.5	67.3
	≥ 10	89.0	82.3
	≥ 40	76.8	99.3
	≥ 160	51.8	100.0

[a] Sensitivity is the proportion of NPC positive to a marker
[b] Specificity is the proportion of non-NPC controls negative to a marker

At the cut-off titre of ≥10 for IgA/VCA, 137 out of 164 NPC sera (83.6%) were positive with GMT of 55.6, while 2.7% controls were positive. GMT for IgG/VCA was 576.5 with 89.0% NPC sera positive. Two percent (3 out of 147) controls were positive for the cut-off titre of ≥160. For IgA/EA, 75.0% NPC sera were positive (GMT=29.0) at the cut-off titre of ≥5 and all the controls were negative. For IgG/EA, 76.8% NPC sera were positive for titre range of 40-2560 with GMT of 166.3. In contrast, only 0.7% controls were positive for the above range. (Table 3.13).

Table 3.13: Percentage of positivity and GMT of EBV serological markers in NPC and non-NPC controls

		NPC (n = 164)	Controls (n = 147)
IgA/VCA	% (+)	83.6 (137/164)	2.7 (4/147)
	GMT *(+)	55.6	10
	Range (+)	10 – 1280	10
IgG/VCA	% (+)	89.0 (146/164)	2.0 (3/147)
	GMT* (+)	576.5	160
	Range (+)	160 – 10240	160
IgA/EA	% (+)	75.0 (123/164)	0 (0/147)
	GMT* (+)	29.0	
	Range (+)	5 – 1280	
IgG/EA	% (+)	76.8 (126/164)	0.7 (1/147)
	GMT* (+)	166.3	40
	Range (+)	40 – 2560	40

*GMT of samples titre at and above the cut-off level

3.5 Comparison of Anti-EBV Serological Markers

IgG/VCA, being the most sensitive marker was used as the reference for comparison to the other 3 markers. Correlation was calculated based on Spearman's R correlation coefficient (r) (Appendix 5) to compare IgG/VCA with the other 3 markers in NPC patients. There were positive correlation with significant correlation between the IgG/VCA and the other markers (Figure 3.16, 3.17 and 3.18).

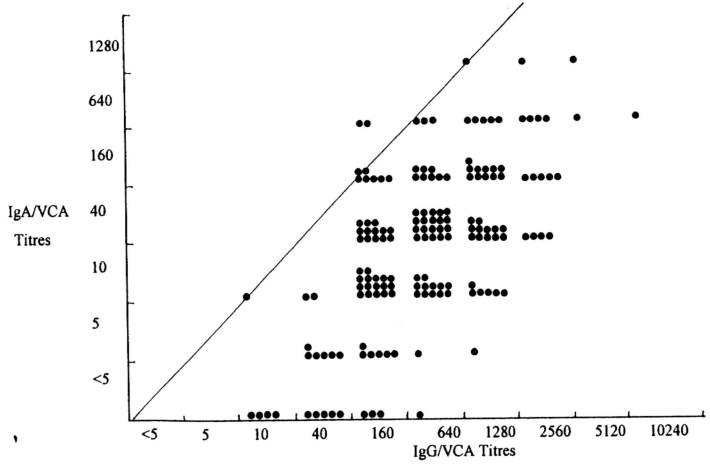

Figure 3.16: IgG/VCA and IgA/VCA titres in NPC samples.
Each dot represents the result of one serum.
Correlation coefficient(r) = 0.61, significantly correlated ($p < 0.01$)

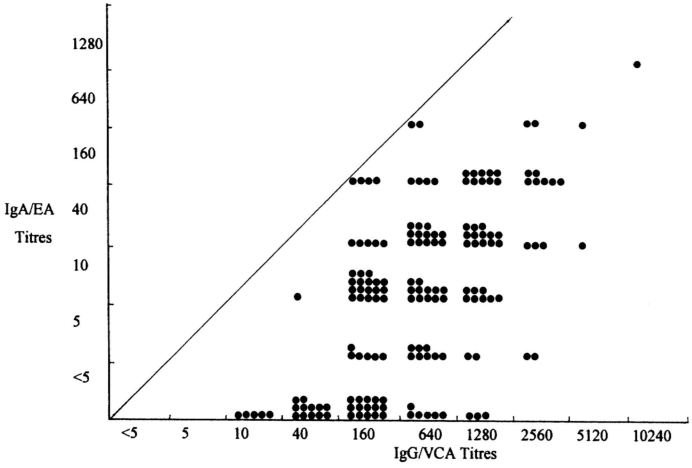

Figure 3.17: IgG/VCA and IgA/EA titres in NPC samples.
Each dot represents the result of one serum.
Correlation coefficient(r) = 0.59, significantly correlated ($p < 0.01$)

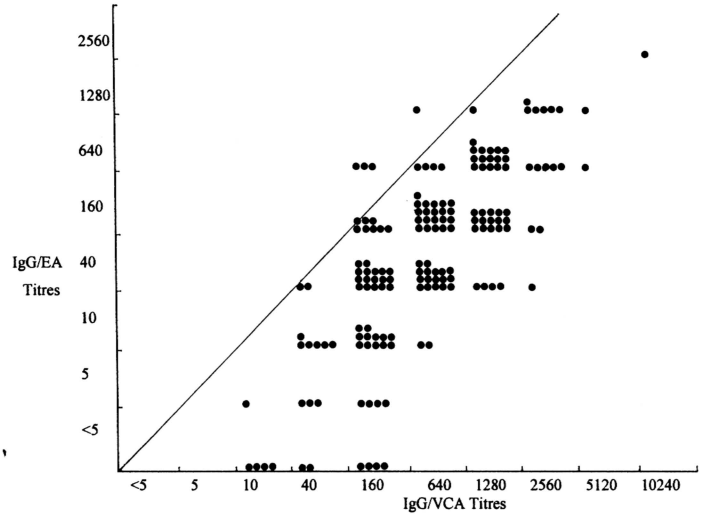

Figure 3.18: IgG/VCA and IgG/EA titres in NPC samples.
Each dot represents the result of one serum.
Correlation coefficient(r) = 0.76, significantly correlated ($p < 0.01$)

3.6 Anti-EBV Serological Profiles

Using the cut-off titre of ≥10 for IgA/VCA, ≥160 for IgG/VCA, ≥5 for IgA/EA and ≥40 for IgG/EA respectively, a serological profile was obtained (Table 3.14). Of the 164 NPC sera tested, 112 sera (68.3%) were positive for all 4 anti-EBV markers. 11.0% (18 sera) were positive for 3 markers. Out of the 18 sera, IgA/VCA, IgG/VCA and IgA/EA antibodies were elevated in 11 sera, and IgA/VCA, IgG/VCA and IgG/EA were elevated in the rest.

Ten sera (6.1%) were positive for only 2 markers. Five sera possessed both IgA and IgG VCA antibodies while the other five had IgG antibodies to EA and VCA. Two sera were positive for only IgA/VCA. Only 1 serum was positive for IgG/EA alone and 7 sera (4.3%) were positive for only IgG/VCA antibodies. Fourteen out of the 164 sera (8.5%) tested were negative for all four markers.

Of the 164 NPC sera tested, 140 sera (85.3%) were positive for at least 2 of the anti-EBV antibodies (Table 3.14). The sensitivity and specificity of various combinations of 2 anti-EBV antibodies were compared in Table 3.15. The sensitivity was calculated from the NPC group for cases positive for both or either marker. Specificity was based on the non-NPC controls cases negative for both markers.

Table 3.14: Anti-EBV serological profiles of NPC samples

	Marker	Number of sera	Percentage (%)
Positive for all 4 markers	IgA/VCA IgG/VCA IgA/EA IgG/EA	112	68.3
Positive for only 3 markers	IgA/VCA IgG/VCA IgA/EA	11	6.7
	IgA/VCA IgG/VCA IgG/EA	7	4.3
Positive for only 2 markers	IgA/VCA IgG/VCA	5	3.0
	IgG/VCA IgG/EA	5	3.0
Positive for only 1 marker	IgA/VCA	2	1.2
	IgG/VCA	7	4.3
	IgG/EA	1	0.6
Negative for all 4 markers		14	8.5
Total		164	100.0

Table 3.15: Sensitivity and specificity of different combinations of 2 anti-EBV antibodies

Combination of markers	Sensitivity (%)	Specificity (%)
IgA/VCA with IgA/EA	83.6	97.3
IgA/VCA with IgG/EA	87.2	96.6
IgA/VCA with IgG/VCA	90.9	95.2
IgG/VCA with IgA/EA	89.6	98.0
IgG/VCA with IgG/EA	90.2	96.3
IgA/EA with IgG/EA	82.9	99.3

3.7 Multipin Peptide Synthesis of EBV BHRF1 Protein

Forty-six 10-residues peptides with peptide spacing increment of 4 amino acids and 8-residue peptides of positive (PLAQ) and negative (GLAQ) controls were synthesised onto the multipin peptide synthesis pins. The peptides were synthesised in duplicates.

3.7.1 Multipin BHRF1 peptide ELISA

A conjugate test was carried out before the peptides were used to test sera samples. This was to ensure that reactivity was not due to background reactions of the pin bound peptides as no primary antibody was used during conjugate testing.

The conjugate test results gave a mean absorbance of 0.0435 at 450/630 nm and was compared with the mean absorbance of 5 NPC sera (Figure 3.19). Therefore, it was cleared that the reactivity was not due to background reactions.

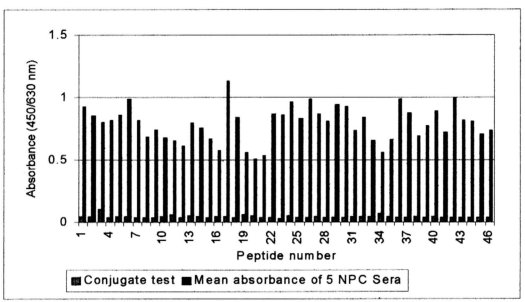

Figure 3.19: Comparison of the mean absorbance between the conjugate test and 5 NPC sera.

3.7.2 IgA linear epitope of BHRF1 in NPC

The reactions of the synthesised control pins were acceptable when compared with pre-synthesised control peptide pins supplied by the manufacturer. The control monoclonal antibody provided bound to positive control (PLAQ) but not negative control (GLAQ) pins. Absorbance at 450/630 nm values for the synthesised control pins averaged 0.625 for PLAQ and 0.386 for GLAQ.

The 46 synthesised overlapping peptides of the BHRF1 protein were tested for the presence of IgA antibodies in 5 NPC sera and 1 control serum using the Multipin ELISA procedure. These NPC sera had elevated IgA/EA titre (determined by immunofluorescence assay). For each peptide, an average absorbance of the duplicate peptide was obtained with every sample. Figure 3.20 shows the absorbance obtained when the 46 overlapping 10 residues BHRF1 peptides were tested with the sera samples. Data of individual serum reacted with the peptides are shown in Figure 3.21 and 3.22. In each individual graphs, the absorbance (450/630 nm) was plotted for each of the 10-mer peptides indicated by peptide number.

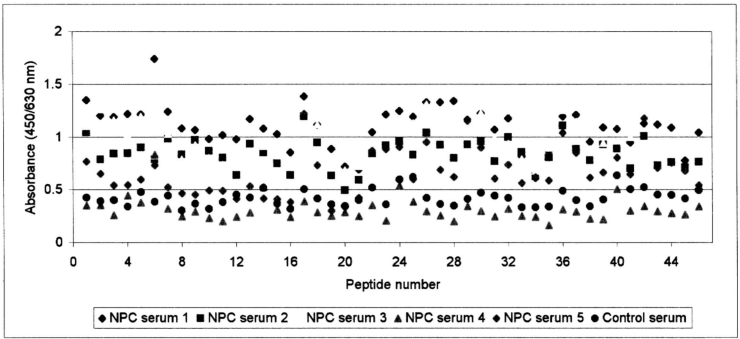

Figure 3.20: BHRF1 peptide reactivity profile for 5 NPC sera and 1 control serum.
Data showed IgA epitope binding patterns. All 5 NPC sera were IgA/EA positive for IFA.

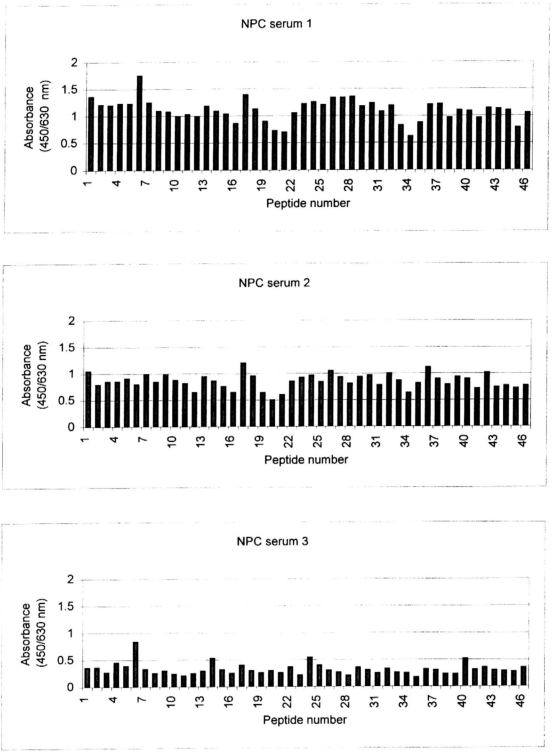

Figure 3.21: BHRF1 peptide reactivity profile of IgA antibodies in 3 NPC sera.

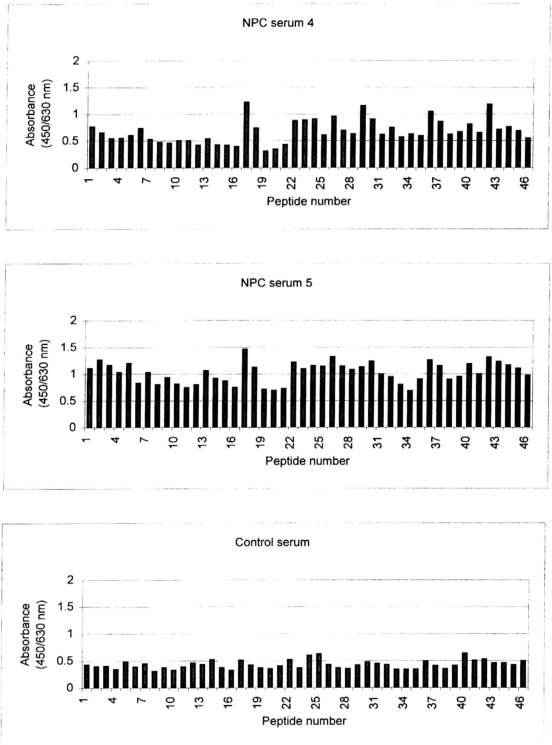

Figure 3.22: BHRF1 peptide reactivity profile of IgA antibodies in 2 NPC sera and 1 control serum.

The NPC sera generally show higher peptide reactivity profile compared to the control. However, there is a high background and the difference between the absorbance of each peptide was small, making the identification of definite peaks difficult. The specific antibody binding for each peptide was calculated by substracting the absorbance of the control serum from the mean absorbance of all the NPC sera as shown in Figure 3.23.

Major reactive BHRF1 regions were observed at peptides 6, 17, 26, 27 and 29. Table 3.16 showed the recognition of the peptides. Peptide 17 is the most recognised peptide with the highest absorbance value and recognising 4 out of 5 NPC sera.

Table 3.16: Major EBV BHRF1 peptides reactive with IgA in NPC sera

Peptide number	Amino acid sequence	Serum with peak observed
6	GNGTLHPVLE	NPC serum 1
		NPC serum 3
17	FTETWNRFIT	NPC serum 1
		NPC serum 2
		NPC serum 4
		NPC serum 5
26	ALAWMAWCMH	NPC serum 1
		NPC serum 2
		NPC serum 5
27	MAWCMHACRT	NPC serum 1
		NPC serum 5
29	RTLCCNQSTP	NPC serum 4

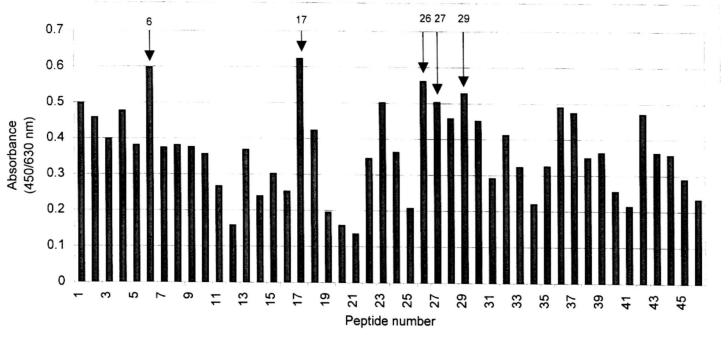

Figure 3.23: The specific IgA antibody binding to BHRF1 peptides in NPC sera.
Bars indicate the mean absorbance of all the NPC sera substracted by the absorbance of the control serum.